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Cold-Shock Regulation of the Arabidopsis *TCH* Genes and the Effects of Modulating Intracellular Calcium Levels[1]

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The Arabidopsis *TCH* genes, which encode calmodulin-related proteins and a xyloglucan endotransglycosylase, are shown to be up-regulated in expression following cold shock. We investigated a possible role of fluctuations in intracellular calcium ion concentrations ($[Ca^{2+}]$) in the cold-shock-induced *TCH* gene expression. Transgenic plants harboring the apoaequorin gene were generated to monitor $[Ca^{2+}]$ and to test the necessity of cold-induced $[Ca^{2+}]$ increases for *TCH* expression. Cold-shock-induced $[Ca^{2+}]$ increases can be blocked by La^{3+} and Gd^{3+}, putative plasma membrane Ca^{2+} channel blockers, and 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, an extracellular Ca^{2+} chelator. Cold-shock-induced expression of the *TCH* genes is inhibited by levels of La^{3+}, Gd^{3+}, and 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, that have been shown to block $[Ca^{2+}]$ increases. These data support the hypotheses that (a) intracellular $[Ca^{2+}]$ increases following cold shock require extracellular Ca^{2+} and may derive from a Ca^{2+} influx mediated by plasmalemma Ca^{2+} channels, and (b) cold up-regulation of expression of at least a subset of the *TCH* genes requires an intracellular $[Ca^{2+}]$ increase. The inhibitors are also shown to have stimulus-independent effects on gene expression, providing strong evidence that these commonly used chemicals have more complex effects than generally reported.

Plants sense and respond to various environmental stimuli, which often enables them to acclimate physiologically to the conditions of their local environment. For example, in response to low temperature, Arabidopsis plants undergo changes that enable them to survive subsequent freezing temperatures (Gilmour et al., 1988; Thomashow, 1994). Environmental stimuli such as cold, wind, or light must be transduced into plant cells as second messenger signals, which control cellular responses. Regulation of gene expression is often an early response and an important step toward generating changes in cellular behavior or properties.

In response to various environmental stimuli, the Arabidopsis *TCH* genes are up-regulated in expression (Braam and Davis, 1990; Braam, 1992; Sistrunk et al., 1994; Xu et al., 1995). Originally, the *TCH* genes were isolated as a result of the strong and rapid up-regulation of expression in response to mechanical stimuli such as touch and wind (Braam and Davis, 1990). The *TCH* genes are also induced in expression in plants or cultured cells following stimulation with darkness or heat shock, respectively (Braam and Davis, 1990; Braam, 1992). A possible explanation of how such diverse stimuli lead to a common molecular response is that the same second messenger may be generated in response to these distinct stimuli, and this signaling molecule controls the levels of *TCH* gene expression. Because several of the stimuli that lead to *TCH* gene up-regulation also result in increased cytosolic free $[Ca^{2+}]$ (Knight et al., 1991, 1992, 1993; Braam, 1992; Haley et al., 1995; D.H. Polisensky and J. Braam, unpublished results), it has been postulated that changes in $[Ca^{2+}]$ may be sufficient for *TCH* gene up-regulation of expression (Braam, 1992).

TCH1 encodes an Arabidopsis CaM, and *TCH2* and *TCH3* encode CaM-related proteins (Braam and Davis, 1990; Sistrunk et al., 1994; K.A. Johnson and J. Braam, unpublished results). As a result of stimulus-induced increases in cytosolic free $[Ca^{2+}]$, CaM is believed to bind to Ca^{2+}, becoming activated to interact with and influence the activity of a variety of target enzymes (Klee and Vanaman, 1982; Roberts et al., 1986; Cohen and Klee, 1988; Allan and Hepler, 1989; Roberts and Harmon, 1992). In this way, CaM acts as a major Ca^{2+} receptor in cells and mediates responses to this second messenger. Related but distinct Ca^{2+}-binding proteins such as TCH3 (Sistrunk et al., 1994) may also regulate target enzymes in a $[Ca^{2+}]$-dependent manner, or they may be involved in other $[Ca^{2+}]$-related processes such as Ca^{2+} sequestration. Therefore, levels of free cytosolic Ca^{2+} may control the expression of genes encoding Ca^{2+}-binding proteins, and this regulation may provide a regulatory circuit that ensures the efficacy of Ca^{2+} as a transient second messenger and maintenance of $[Ca^{2+}]$ homeostasis in plant cells.

It is possible that increases in cytosolic $[Ca^{2+}]$ mediate plant responses to cold temperatures (Minorsky, 1985, 1989; Minorsky and Spanswick, 1989). Cold shock results in transient increases in $[Ca^{2+}]$ in tobacco seedlings (Knight et al., 1991), and chilling causes an influx of $^{45}Ca^{2+}$ into *Chara* (Reid and Smith, 1992) and alfalfa protoplasts (Monroy and Dhindsa, 1995). Mechanosensitive-Ca^{2+}-selective cation channels are strongly influenced by temperature (Ding and Pickard, 1993a). Ca^{2+} chelators and channel blockers have been shown to inhibit cold acclimation (Monroy et al., 1993) and expression of *cas15* and *cas18*, two cold acclimation-specific genes (Monroy and Dhindsa, 1995), in alfalfa suspension cell cultures.

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Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; CaM, calmodulin.

Because of the strong data linking cold temperature responses in plants to changes in $[Ca^{2+}]$, we investigated whether cold shock induces changes in intracellular $[Ca^{2+}]$ and *TCH* gene expression in Arabidopsis. Our approach for monitoring intracellular $[Ca^{2+}]$ increases in Arabidopsis takes advantage of the technology developed by Knight et al. (1991). These researchers showed that expression of the jellyfish gene encoding the $[Ca^{2+}]$-dependent apoaequorin protein in tobacco plants can result in detectable luminescence when intracellular Ca^{2+} levels increase. Here, we report that aequorin transgenic Arabidopsis plants, like tobacco, emit light when cold-shocked, indicating that cold stimulation causes intracellular $[Ca^{2+}]$ increases. Cold-shock-induced $[Ca^{2+}]$ increases can be inhibited by Ca^{2+} chelators and putative channel blockers that are thought to act at the cell exterior, indicating that external Ca^{2+} is necessary for the cytosolic $[Ca^{2+}]$ changes. Cold shock results in a strong, rapid, and transient induction of expression of the Arabidopsis *TCH* genes, and the cold-shock-induced $[Ca^{2+}]$ increase may be necessary for the subsequent up-regulation of expression of a subset of the *TCH* genes. However, stimulus-independent effects of commonly used putative Ca^{2+} channel blockers and a Ca^{2+} chelator on *TCH* gene expression indicate that the cellular responses to these chemicals are complex.

MATERIALS AND METHODS

Plant Growth and Manipulations

Plants were maintained at 24°C in 65 to 75% humidity under constant light. To generate transgenic Arabidopsis plants, the pMAQ2 plasmid (generously provided by Marc Knight, Oxford University, UK, and Anthony Trewavas, University of Edinburgh, UK; Knight et al., 1991) containing the apoaequorin gene was electroporated into *Agrobacterium tumefaciens* LBA4404. *Arabidopsis thaliana*, ecotype RLD, was transformed according to the method of Valvekens et al. (1988). Analyses were performed on third-generation plants that were homozygous for kanamycin resistance. This seed is available from the Arabidopsis Biological Resource Center at The Ohio State University (seeds@genesys.cps.msu.edu). Cultured cells were generated as described previously (Braam, 1992).

Cold-Shock Stimulation

To monitor intracellular $[Ca^{2+}]$ fluctuations in whole plants, soil-grown seedlings were gently rinsed with water, placed between microscope slides and coverslips, and hydrated with water. The plants were allowed to recover from the manipulations in the dark overnight. The coelenterazine lumiphore (a generous gift from Molecular Probes, Eugene, OR) was applied at 2.5 μM under the coverslip, incubation was continued for 6 to 18 h, and cold shock was administered by placing the slides on the surface of a 0°C metal block for 2 min. The slides were placed immediately onto autoradiographic film.

All other cold-shock treatments were performed on cultured cells. Cells were maintained in 1× Gamborg's B5 media (Sigma), 34.2 g/L Suc, 0.5 mg/L 2–4-D (Sigma), and 0.05 mg/L kinetin (Sigma), adjusted to pH 5.8 with KOH. However, to prevent precipitate formation in the presence of $LaCl_3$ and $GdCl_3$, B5 medium that lacked phosphate ("modified medium") was used during the experimental procedures. For luminescence detection, approximately 50 mg of cells were aliquoted into each 2-mL Eppendorf tube containing 400 μL of modified medium, and transferred to a rotator in the dark at room temperature for 12 to 16 h. The medium was decanted and replaced with 400 μL of modified medium containing 2 μM coelenterazine, and incubated for 2 h on a rotator in the dark. Fifteen minutes prior to cold-shock treatment, the Eppendorf tubes were removed from the rotator and the cells were allowed to settle. The basal fluorescence level was determined with a TD-20e Luminometer (Turner Designs, Sunnyvale, CA) over 10 s. Cold shock was administered by gentle immersion of the Eppendorf tubes in an ice water bath for 2 min, and the luminescence was read again immediately. Luminescence values were determined by subtraction of the control luminescence reading from the cold-shock experimental reading.

For RNA analysis, cells were maintained in flasks containing 10 mL of medium. For cold-shock treatment, the flasks were gently swirled in ice water for 2 min to rapidly bring the temperature of the medium to 0°C. The flasks were then swirled in water at room temperature for 2 min and returned to a rotary shaker at 120 rpm for the indicated times. Control cells were treated exactly as described, except the first 2-min incubation was conducted in a water bath at room temperature.

When indicated, $LaCl_3$, $GdCl_3$, Mes, BAPTA, and/or $CaCl_2$ were added to the incubation medium 2 h prior to cold shock. To test the reversibility of the inhibitor effects, the 2-h incubation was followed by removal of the medium containing inhibitor and several washes in fresh medium during a 2-h period. Cells were then incubated an additional 2 h with coelenterazine before cold-shock stimulation and luminescence detection. Control samples were treated identically during the 6-h period except that no inhibitors were present during the first incubation.

Northern Blot Analysis

For RNA gel blots, total RNA was purified (Verwoerd et al., 1989), subjected to electrophoresis on formaldehyde gels, and transferred to filters. Filters were probed with hexamer-labeled DNA fragments (Feinberg and Vogelstein, 1983). Probes were derived from partial *TCH* gene cDNAs (Braam and Davis, 1990) and a *Bam*HI-*Kpn*I fragment of an Arabidopsis β-tubulin (Marks et al., 1987).

RESULTS

Aequorin Luminescence Reports Cold-Shock-Induced $[Ca^{2+}]$ Increases in Transgenic Arabidopsis

To enable monitoring of intracellular $[Ca^{2+}]$ changes, we generated transgenic Arabidopsis plants that harbor a cDNA encoding the *Aequorea victoria* apoaequorin protein (Knight et al., 1991) under the control of the relatively constitutive cauliflower mosaic virus 35S promoter. We

detected cold-shock-induced intracellular [Ca^{2+}] increases in these transgenic Arabidopsis plants by measuring luminescence in two different ways. First, as shown in Figure 1, aequorin luminescence in Arabidopsis was of sufficient magnitude to expose autoradiographic film. When transgenic Arabidopsis plants with reconstituted aequorin were cold-shocked, they luminesced throughout most of the shoot (Fig. 1B). Luminescence from the petioles was particularly strong, and we did not detect luminescence from the root. This could be because the cold-shock-induced [Ca^{2+}] increases were restricted to shoot tissue, the aequorin protein did not accumulate to sufficient quantities in the root, or the roots were too small to emit sufficient quantities of light. Under similar conditions and at the same duration of autoradiographic film exposure, unstimulated transgenic plants showed no response (Fig. 1A).

Cold-shock-induced intracellular [Ca^{2+}] increases in cultured cells were also assayed using a luminometer. As shown in Figure 2, A and B (bars labeled "0"), within 10 s of a 2-min 0°C cold shock, a significant increase in aequorin-generated luminescence was apparent. Although we consistently detected a significant luminescence in response to cold shock and the response level was consistent within each experiment, distinct experiments yielded increases of luminescence between 100- and 10,000-fold over background (data not shown). We suspect that some of this variability was due to the age or quality of the coelenterazine lumiphore. These results indicate that a cold-shock stimulus results in immediate and strong intracellular [Ca^{2+}] increases that can be monitored using the Ca^{2+}-

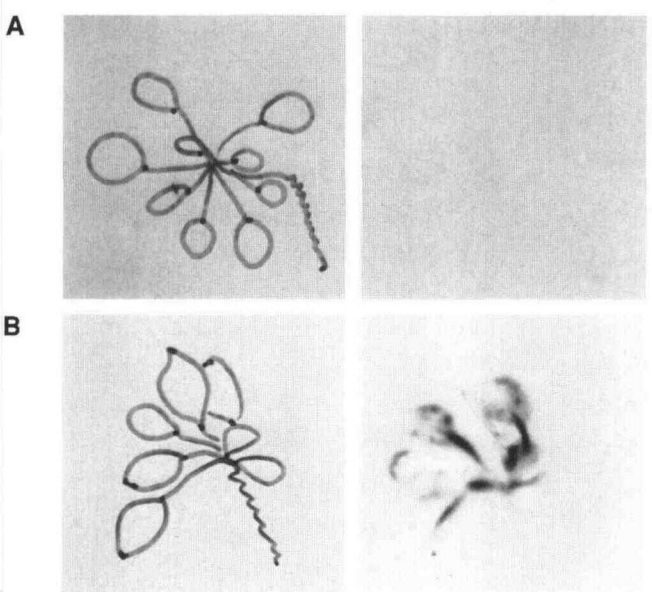

Figure 1. Cold shock results in intracellular [Ca^{2+}] increases visualized as aequorin luminescence of Arabidopsis seedlings. Transgenic Arabidopsis seedlings expressing the gene encoding aequorin were soaked with the coelenterazine lumiphore and then exposed to room temperature (A) or cold-shocked (B). Aequorin luminescence is detected as blackness on autoradiographic film (right). Exposure times were approximately 16 h and were identical for A and B. Outlines of the seedlings are shown at left.

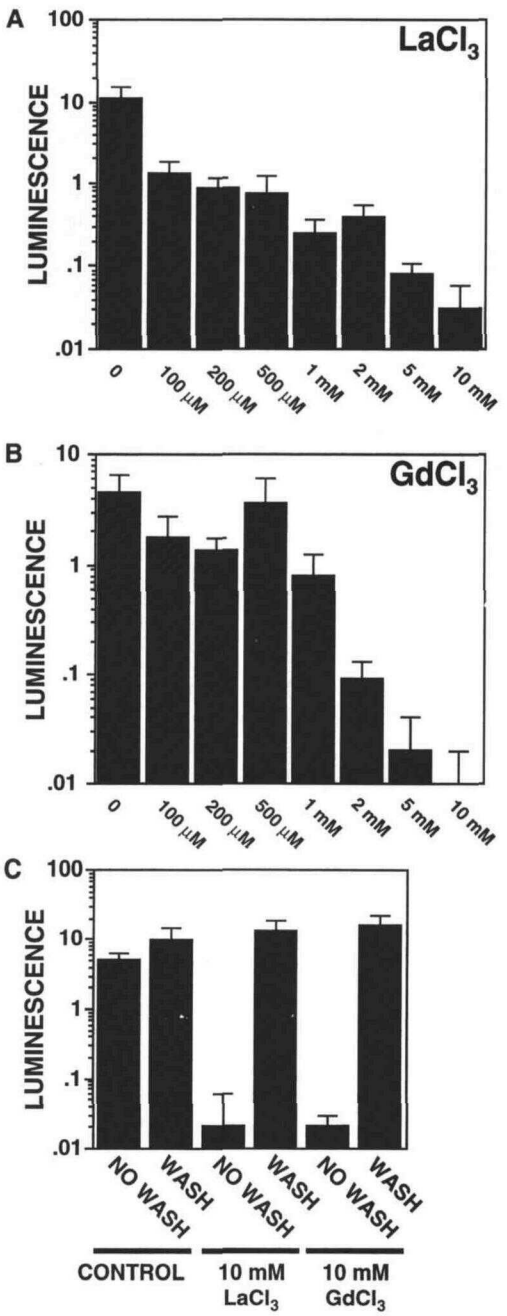

Figure 2. Cold-shock-induced intracellular Ca^{2+} increases are blocked by La^{3+} and Gd^{3+}. Increases in luminescence readings were recorded from cold-shocked, transgenic aequorin Arabidopsis cultured cells after preincubation with coelenterazine in the absence of inhibitor (0) or in the presence of indicated concentrations of $LaCl_3$ (A) or $GdCl_3$ (B). In C, cells were treated as in A and B (no wash), or after the incubation with or without inhibitor as indicated, and the cells were washed with medium lacking inhibitor for 2 h prior to assaying cold-shock-induced increases in luminescence (wash). Data from five independent samples with SD values are represented in each bar.

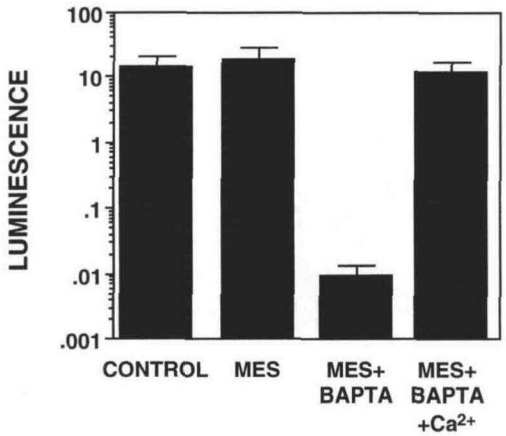

Figure 3. Cold-shock-induced intracellular Ca^{2+} increases are blocked by BAPTA. Increases in luminescence readings were recorded from cold-shocked, transgenic aequorin Arabidopsis cultured cells after preincubation with coelenterazine without additions (Control); with 2 mM Mes (Mes); with 2 mM Mes and 10 mM BAPTA (Mes + BAPTA); or with 2 mM Mes, 10 mM BAPTA, and 11 mM $CaCl_2$ (Mes + BAPTA + Ca^{2+}). Data from five independent samples with SDs are represented in each bar.

dependent luminescence of aequorin in transgenic Arabidopsis plants or cultured cells.

Cold-Shock-Induced Intracellular [Ca^{2+}] Increases Require Extracellular Ca^{2+}

Knight et al. (1992) reported that high concentrations (10 mM) of the putative plasma membrane Ca^{2+} channel blockers La^{3+} and Gd^{3+} can block cold-shock-induced [Ca^{2+}] increases in tobacco plants. As shown in Figure 2, A and B, a range of concentrations of either La^{3+} or Gd^{3+} significantly inhibited the cold-shock-induced [Ca^{2+}] increase in Arabidopsis cultured cells, with 10 mM levels blocking the [Ca^{2+}] increase most effectively. To ensure that the high concentrations of inhibitor were not lethal or did not otherwise permanently affect the ability of cells to respond, we tested whether the effects of 10 mM La^{3+} and Gd^{3+} were reversible. When the inhibitors were washed out of the medium after the 2-h incubation with the cells, the ability of the cells to respond to cold shock was restored (Fig. 2C) and the cells continued to grow (data not shown); therefore, inhibition of the cold-shock-induced [Ca^{2+}] increase by Gd^{3+} and La^{3+} is reversible and is not a consequence of permanent disability or death of the cells. Thus, the cold-shock-induced [Ca^{2+}] increase in Arabidopsis, like tobacco (Knight et al., 1992), can be blocked by putative plasma membrane Ca^{2+} channel blockers.

Because of potential nonspecific effects from the use of high concentrations of La^{3+} and Gd^{3+}, we wanted to block the putative cold-shock-induced influx of Ca^{2+} in another manner. We used the Ca^{2+} chelator BAPTA (Tsien, 1980), which is believed to remain extracellular, to reduce the availability of apoplastic Ca^{2+}. Monroy and Dhindsa (1995) recently reported that preincubation in BAPTA blocks uptake of $^{45}Ca^{2+}$ in alfalfa protoplasts following chilling at 4°C. Strong preferential chelation of Ca^{2+} with BAPTA requires a pH of greater than 6.5 (Tsien, 1980); therefore, we incubated cells in Mes buffer at neutral pH prior to incubation with BAPTA. Incubation in Mes alone had no significant effect on cold-shock-induced [Ca^{2+}] increases (Fig. 3). In the presence of BAPTA, however, the luminescence signal was decreased by three orders of magnitude (Fig. 3). This inhibition of the intracellular [Ca^{2+}] increase was most likely due to specific chelation of extracellular Ca^{2+}, because provision of excess Ca^{2+} restores the robust cold-shock-induced intracellular [Ca^{2+}] increase (Fig. 3).

These data obtained using putative Ca^{2+} channel blockers and a Ca^{2+} chelator are largely consistent with those reported from work on other plant species (Knight et al., 1992; Monroy and Dhindsa, 1995). The results using BAPTA chelation indicate that the cold-shock-induced increase in intracellular [Ca^{2+}] in Arabidopsis cells requires availability of external Ca^{2+}, and inhibition of [Ca^{2+}] increases by La^{3+} and Gd^{3+} following cold shock is consistent with the possibility that Ca^{2+} flows into the cells through plasma membrane Ca^{2+} channels.

Regulation of *TCH* Gene Expression by Cold Shock

The Arabidopsis *TCH* genes are regulated in expression by various environmental stimuli, several of which are thought to cause cytosolic [Ca^{2+}] increases (Braam, 1992). Therefore, it is possible that fluctuations in cytosolic [Ca^{2+}] control *TCH* gene expression levels. Because cold shock causes an immediate increase in cytosolic [Ca^{2+}], we tested whether *TCH* gene expression is up-regulated by cold shock. Figure 4A shows that, following a transient cold shock of 0 or 5°C, there was a very significant increase in *TCH* mRNA levels. Expression of the *TCH* genes was not

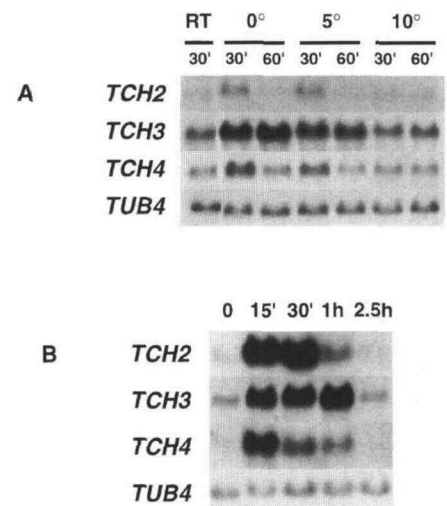

Figure 4. Cold-shock induction of *TCH* gene expression. A, Arabidopsis cultured cells were subjected to a 2-min room temperature treatment (RT) or to cold shock at the indicated temperatures (°C). Cells were then harvested after either 30 or 60 min as indicated. RNA was purified, subjected to formaldehyde gel electrophoresis, blotted to filters, and hybridized with probes listed at left. B, Arabidopsis cultured cells were left unstimulated (0) or subjected to a 2-min 0°C cold shock and harvested at the times shown. ', min; h, hour.

significantly affected by a transient, 10°C temperature shock. The cold-shock-induced *TCH* gene response was fast and transient; *TCH* mRNAs increased within 15 min of 0°C stimulation and returned to basal levels within 2.5 h (Fig. 4B).

Effect of Ca^{2+} Channel Blockers and a Ca^{2+} Chelator on *TCH* Gene Expression

Our data indicate that cold shock of Arabidopsis leads to intracellular [Ca^{2+}] increases and to *TCH* gene up-regulation of expression. We wanted to determine whether these two cellular responses are functionally linked. Therefore, we investigated whether the cold-shock-induced cytosolic [Ca^{2+}] increases were necessary for cold-shock up-regulation of *TCH* gene expression. When Arabidopsis cultured cells were preincubated with La^{3+} and Gd^{3+} levels shown to strongly block the cold-shock-induced intracellular [Ca^{2+}] increases (Fig. 2), we found that cold-shock-induced *TCH* gene expression was significantly inhibited (Fig. 5, A and B). Lower concentrations of inhibitors that partially reduce the cold-shock-induced [Ca^{2+}] increase had reduced or no effect on the induction of *TCH* gene expression. One interpretation of this result is that even moderate increases in cytosolic [Ca^{2+}] are sufficient to regulate *TCH* gene expression.

Surprisingly, *TCH* mRNA accumulation levels could be enhanced at lower concentrations of La^{3+}. For example, *TCH4* mRNA levels were higher in cells cold-shocked in the presence of 500 μM to 2 mM La^{3+} than in cells cold-shocked in the absence of La^{3+}. Indeed, incubation of cells with La^{3+} or Gd^{3+} alone was sufficient to up-regulate expression of *TCH3* and *TCH4*, even in the absence of cold shock (Fig. 5, C and D); *TCH2* expression may also have been weakly affected (Fig. 5, C and D). This induction of gene expression was highest at 1 to 5 mM La^{3+} and Gd^{3+}, whereas in the presence of 10 mM inhibitors, *TCH* gene expression levels were close to basal levels. The mechanism of this cold-shock-independent response of the *TCH* genes to La^{3+} and Gd^{3+} is not clear. It is possible that up-regulation of *TCH* gene expression results as a consequence of either plasma membrane Ca^{2+} channel inhibition or other potential effects of these compounds that are distinct from their proposed roles in Ca^{2+} channel blockage (see "Discussion").

Figure 6 shows that steady-state and induced expression levels of the *TCH* genes were not significantly affected by incubation of cells in Mes buffer alone. In the presence of BAPTA, however, cold shock no longer resulted in the induction of expression of *TCH2* and *TCH4*. This inhibition of expression was most likely due to specific chelation of

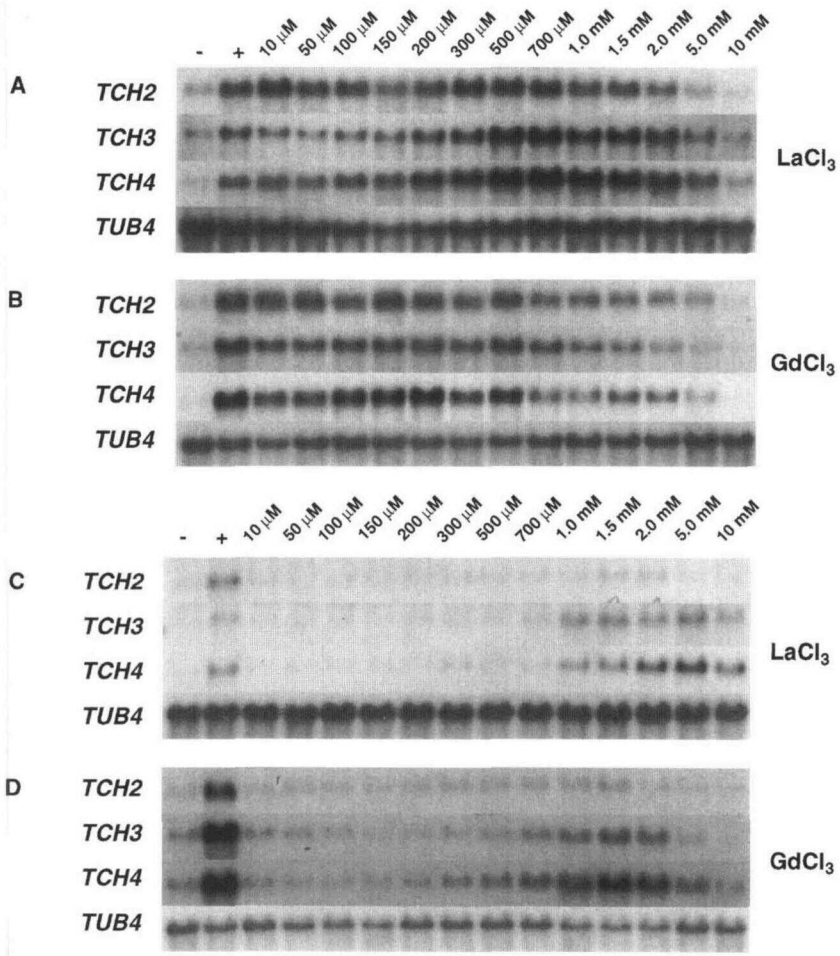

Figure 5. Effects of La^{3+} and Gd^{3+} on *TCH* gene expression. Arabidopsis cultured cells were incubated for 2 h in the absence of inhibitors and left unstimulated (−) or cold-shocked at 0°C for 2 min (+). Cells were cold-shocked (A and B) or left unstimulated (C and D) after a 2-h incubation with the indicated concentrations of $LaCl_3$ (A and C) or $GdCl_3$ (B and D). Cells were harvested after 30 min, and RNA was purified, subjected to formaldehyde gel electrophoresis, blotted to filters, and hybridized with the probes listed at left.

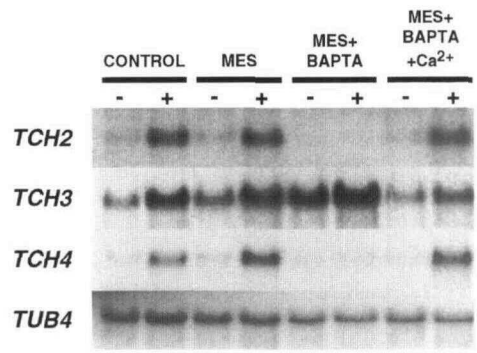

Figure 6. Effects of BAPTA on *TCH* gene expression. Arabidopsis cells were left unstimulated (−) or subjected to a 2-min 0°C cold shock (+) after a 2-h incubation with no additions (CONTROL); 2 mM Mes (Mes); 2 mM Mes and 10 mM BAPTA (Mes + BAPTA); or 2 mM Mes, 10 mM BAPTA, and 11 mM $CaCl_2$ (Mes + BAPTA + Ca^{2+}). Cells were harvested after 30 min, and RNA was purified, subjected to formaldehyde gel electrophoresis, blotted to filters, and hybridized with the probes listed at left.

Ca^{2+} by BAPTA, because up-regulation of expression of these genes is restored when excess Ca^{2+} is provided. Steady-state level expression of *TCH3* was increased in cells incubated in BAPTA alone, and administration of a cold shock resulted in a slight increase of the *TCH3* mRNA levels. Cells incubated in BAPTA and excess Ca^{2+} showed normal levels of *TCH3* expression, and cold-shock up-regulation of expression was at least partially restored. These data suggest that the availability of external Ca^{2+} is necessary for cold-shock induction of expression of the *TCH* genes; however, chelation of external Ca^{2+} alone, even in the absence of cold shock, was sufficient to perturb *TCH3* expression.

DISCUSSION

Cold Shock Induces Intracellular Ca^{2+} Increases in Arabidopsis That Can Be Blocked by La^{3+}, Gd^{3+}, or BAPTA

As predicted (Minorsky, 1985, 1989; Minorsky and Spanswick, 1989; Ding and Pickard, 1993b; Monroy and Dhindsa, 1995) and as shown for tobacco (Knight et al., 1991), cold-shock stimulation of Arabidopsis plants or cultured cells leads to intracellular [Ca^{2+}] increases. This response can be monitored using Ca^{2+}-dependent luminescence of aequorin reconstituted in Arabidopsis plants (or cells) engineered to express an apoaequorin transgene. The cold-shock-induced intracellular [Ca^{2+}] increase is strong (up to a 10,000-fold increase in luminescence) and can be detected generally throughout the shoot.

Inhibition of the cold-shock-induced [Ca^{2+}] increase by an extracellular Ca^{2+} chelator indicates that external Ca^{2+} is a requirement for the cold-shock-induced intracellular [Ca^{2+}] increase. Incubation of cells with millimolar concentrations of La^{3+} and Gd^{3+}, putative plasma membrane Ca^{2+} channel blockers, also largely prevents the cold-shock-induced [Ca^{2+}] increase, suggesting that influx may normally occur through these channels. These results and interpretations are consistent with published reports of experiments in which these same chemicals were used to block cold responses of plants (Knight et al., 1992; Monroy and Dhindsa, 1995). However, the unexpected behavior of *TCH* gene expression in cells exposed to these chemicals, as discussed later, suggests that La^{3+}, Gd^{3+}, and BAPTA have other effects that may or may not be directly related to their putative roles in interfering with Ca^{2+} channel activity or external Ca^{2+} availability.

Expression of the Arabidopsis *TCH* Genes is Up-Regulated by Cold Shock

Cold-shock stimulation also results in a rapid, strong, and transient increase in expression of the Arabidopsis *TCH2*, *TCH3*, and *TCH4* genes. The kinetics and magnitude of up-regulation are similar to *TCH* gene expression following mechanical stimuli (touch and wind), heat shock, and darkness (Braam and Davis, 1990; Braam, 1992; Sistrunk et al., 1994). The mechanism(s) by which these distinct stimuli result in the common molecular response of *TCH* gene regulation is unknown. However, evidence has accumulated that suggests that cytosolic [Ca^{2+}] increases may be the signal that serves to regulate *TCH* gene expression: (a) *TCH2* and *TCH3* encode proteins closely related to a major Ca^{2+} receptor, CaM (Braam and Davis, 1990; Sistrunk et al., 1994; K.A. Johnson and J. Braam, unpublished data); (b) several of the stimuli known to result in *TCH* gene up-regulation of expression, such as touch, wind, wounding, and cold shock, elicit intracellular [Ca^{2+}] increases (Knight et al., 1991, 1992, 1993; Braam, 1992; Haley et al., 1995; D.H. Polisensky and J. Braam, unpublished results); and (c) increases in extracellular [Ca^{2+}] are sufficient to up-regulate *TCH* gene expression (Braam, 1992). With this work, we attempted to determine whether the intracellular increases in [Ca^{2+}] are necessary for the cold-shock-induced *TCH* gene expression. These experiments provide evidence that, although a [Ca^{2+}] increase may be necessary for up-regulation of *TCH* gene expression, there are other effects caused by a Ca^{2+} chelator and Ca^{2+} channel modulators that are not apparent when simply monitoring overall [Ca^{2+}], and that can significantly alter *TCH* gene expression levels.

Regulation of *TCH* Gene Expression in the Presence of La^{3+}, Gd^{3+}, and BAPTA

There is evidence that La^{3+} and Gd^{3+} both inhibit plasma membrane Ca^{2+} channels in addition to other ion channels (reviewed by Bush, 1995). We found that La^{3+} is more efficient at moderate concentrations than Gd^{3+} in reducing the cold-shock-induced increase in intracellular [Ca^{2+}] (Fig. 2, A and B). For example, at 1 mM La^{3+}, there is a 40-fold reduction in the cold-shock-induced increase in aequorin luminescence, whereas at the same concentration of Gd^{3+} the reduction is less than 6-fold. In contrast, significant inhibition of cold-shock regulation of *TCH* gene expression occurs at 1 mM Gd^{3+}, whereas 1 mM La^{3+} is not inhibitory (Fig. 5, A and B). One possible explanation for this discrepancy is that La^{3+} and Gd^{3+} block distinct chan-

nels and that the Ca^{2+} transported through the Gd^{3+}-sensitive channels plays a more critical role in cold-shock-induced regulation of *TCH* gene expression. Mechanosensitive channels are more strongly inhibited by Gd^{3+} than La^{3+} (Ding and Pickard 1993a), and thus these channels, which also are strongly influenced by temperature, may play important roles in cold signal transduction in plants (Ding and Pickard, 1993b).

Interpretation of the effects of La^{3+} and Gd^{3+} on the cold-shock-induced up-regulation of *TCH* gene expression is complicated by the fact that these ions significantly up-regulate expression of *TCH3* and *TCH4* in otherwise unstimulated cells (Fig. 5, C and D). Similarly, BAPTA affects steady-state levels of *TCH3* expression (Fig. 6). These effects on *TCH* gene expression could be indirect. If expression of the *TCH* genes is up-regulated by increased cytosolic $[Ca^{2+}]$ as hypothesized (Braam, 1992), then it is theoretically possible that these chemicals modulate *TCH* gene expression levels (in the absence of other inducing stimuli) by causing cytosolic $[Ca^{2+}]$ increases. Chelation of external Ca^{2+} or blockage of Ca^{2+} influx across the plasma membrane may reduce cytosolic $[Ca^{2+}]$, and to maintain $[Ca^{2+}]$ homeostasis, Ca^{2+} may be released from internal stores. It is also possible that La^{3+} and Gd^{3+} could compete for Ca^{2+} binding to cell walls and thus release bound Ca^{2+}, effectively increasing availability of external Ca^{2+} and possibly cellular uptake. Indeed, although La^{3+} can block plasma membrane Ca^{2+} channels of animal cells, it has also been shown to be capable of enhancing Ca^{2+} influx (Segal, 1986). Although increases in Ca^{2+} flux across internal membranes or the plasma membrane theoretically could explain *TCH* gene up-regulation by these chemicals, we have not detected an overall increase in Ca^{2+}-dependent aequorin luminescence in La^{3+}-, Gd^{3+}-, or BAPTA-treated cells. We cannot rule out, however, that it is the transport of Ca^{2+}, and not the overall levels of cytosolic $[Ca^{2+}]$, that serve to signal *TCH* gene up-regulation.

An alternative explanation for the La^{3+} and Gd^{3+} effects on *TCH* gene expression in unstimulated cells is that, if these ions were entering the cells, they could be substituting for intracellular Ca^{2+}. Although it is commonly believed that La^{3+} and Gd^{3+} are unlikely to enter plant cells, there is evidence to the contrary, at least for Gd^{3+} (Quiquampoix et al., 1990). Furthermore, there is increasing evidence of La^{3+} entry into animal cells (Weihe et al., 1977; Wendt-Gallitelli and Isenberg, 1985; Peeters et al., 1989; Pillai and Bikle, 1992; Powis et al., 1994). Therefore, it is probable that La^{3+} and Gd^{3+} enter plant cells. The effects of La^{3+} and Gd^{3+} on *TCH* gene expression, therefore, may not be mediated through effects on plasma membrane Ca^{2+} channels. Instead, La^{3+} and Gd^{3+} could inhibit other, intracellular ion channels (Bush 1995; Klüsener et al., 1995). In addition, because La^{3+} and Gd^{3+} have crystalline ionic radii similar to that of Ca^{2+} (Weast, 1986), these ions may act intracellularly as Ca^{2+} substitutes. La^{3+} and other lanthanide ions could interact with and influence the activities of cytosolic Ca^{2+}-modulated target proteins, including CaM (for example, see Schultz and Klumpp, 1982; Mulqueen et al., 1985; Buccigross et al., 1986). Lanthanide ions have been shown to activate calpain I and II, which are normally Ca^{2+}-dependent (Zimmerman and Schlaeffer, 1988). Thus, if increases in cytosolic $[Ca^{2+}]$ are sufficient to up-regulate *TCH* gene expression, significant levels of La^{3+} or Gd^{3+} within the cell may substitute for Ca^{2+} and trigger the same responses.

Figure 5, C and D, shows that at the highest levels (10 mM) of La^{3+} and Gd^{3+} the cold-shock-independent up-regulation of *TCH* gene expression is not evident. It is possible that at 10 mM concentrations the inhibitors block entry not only of Ca^{2+} but also of La^{3+} or Gd^{3+} as well. A similar conclusion was made from the data of experiments monitoring La^{3+} uptake through the sodium-calcium exchange pathway of animal cells (Powis et al., 1994) Below 1 mM La^{3+}, La^{3+} uptake was evident; whereas at concentrations greater than 1 mM, La^{3+} influx was inhibited (Powis et al., 1994).

The data presented here indicate that La^{3+} and Gd^{3+} likely affect more than plasma membrane Ca^{2+} channels when added externally to plant cells. It is probable that La^{3+} and Gd^{3+} can be taken up by plant cells and that at least some of their effects may be related to intracellular actions. BAPTA is more likely to remain extracellular, but its effects on the steady-state levels of *TCH3* expression suggest that chelation of extracellular Ca^{2+} can have significant intracellular effects; as yet the mechanism of this activity is unknown.

Significance of Cold-Shock-Induced Expression of the Arabidopsis *TCH* Genes

Several Arabidopsis genes that are up-regulated in expression by cold have been previously identified (reviewed by Thomashow, 1994). A *cis* regulatory sequence involved in cold-regulation of Arabidopsis genes has been identified (White et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). The core sequence, CCGAC, is not found in the 5′ region of the *TCH2*, *TCH3*, or *TCH4* genes (K.A. Johnson, M.L. Sistrunk, W. Xu, and J. Braam, unpublished results). Therefore, cold-shock regulation of gene expression in Arabidopsis likely involves distinct *cis* and *trans* regulatory elements.

Except for alcohol dehydrogenase (Jarillo et al., 1993), the biochemical functions of the products of the Arabidopsis cold-induced genes are not known. Some of the proteins, however, have characteristics consistent with possible roles as cryoprotectants (Thomashow, 1994).

The Arabidopsis *TCH* genes are up-regulated in expression by various environmental stimuli, including touch, wind, darkness, heat shock, and, as shown here, cold shock. This regulation suggests that in response to different stimuli there is a need in the cells for increased synthesis of the *TCH* gene products. For what purpose does Arabidopsis have a common *TCH* gene response to such distinct stimuli? It is possible that there are common physiological responses to all of these different stimuli and the *TCH* gene products are needed to carry out those physiological changes. Alternatively, the *TCH* gene products may have different roles, depending on the requirement. For example, the *TCH2*- and *TCH3*-encoded proteins are closely

related to CaM and therefore may regulate target enzymes in a Ca^{2+}-modulated manner. It is possible that in response to distinct stimuli different proteins are targeted for activation, and thus the appropriate physiological responses are elicited.

Whether the TCH proteins play important roles in the cold response of plants remains to be determined. The finding that an inhibitor of CaM function can inhibit cold acclimation (Monroy et al., 1993) suggests that the functioning of CaM and/or CaM-related proteins, such as TCH2 and TCH3, is critical for cold responses. *TCH4* encodes a xyloglucan endotransglycosylase and can modify the major hemicellulose of the cell wall (Xu et al., 1995); the physiological consequences of this enzymatic activity are not yet clear. However, it is possible that xyloglucan endotransglycosylase-promoted cell wall changes could contribute to cold acclimation and freezing tolerance, as well as to tissue reinforcement in response to mechanical stress. Mechanically stimulated plants are more freeze-tolerant than untreated controls (Jaffe and Biro, 1979), suggesting that some of the physiological responses to mechanical stress are similar to the alterations required for cold tolerance.

Thus, *TCH* gene regulation of expression as a common and rapid response to diverse stimuli could represent one of the earliest steps toward altering physiological properties of plants such that they can survive diverse environmental conditions.

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LITERATURE CITED

Allan E, Hepler PK (1989) Calmodulin and calcium-binding proteins. *In* A Marcus, ed, The Biochemistry of Plants, Vol 15. Academic Press, New York, pp 455–484

Braam J (1992) Regulated expression of the calmodulin-related *TCH* genes in cultured *Arabidopsis* cells: induction by calcium and heat shock. Proc Natl Acad Sci USA **89:** 3213–3216

Braam J, Davis RW (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell **60:** 357–364

Buccigross JM, O'Donnell CL, Nelson DJ (1986) A flow-dialysis method for obtaining relative measures of association constants in calmodulin-metal-ion systems. Biochem J **235:** 677–684

Bush DS (1995) Calcium regulation in plant cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol **46:** 95–122

Cohen P, Klee CB, eds (1988) Calmodulin. *In* Molecular Aspects of Cellular Regulation, Vol 5. Elsevier Biomedical Press, Amsterdam, The Netherlands

Ding JP, Pickard BG (1993a) Mechanosensory calcium-selective cation channels in epidermal cells. Plant J **3:** 83–110

Ding JP, Pickard BG (1993b) Modulation of mechanosensitive calcium-selective cation channels by temperature. Plant J **3:** 713–720

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in *Arabidopsis thaliana*. Plant Physiol **87:** 745–750

Haley A, Russell AJ, Wood N, Allan AC, Knight M, Campbell AK, Trewavas AJ (1995) Effects of mechanical signaling on plant cell cytosolic calcium. Proc Natl Acad Sci USA **92:** 4124–4128

Jaffe MJ, Biro R (1979) Thigmomorphogenesis: the effect of mechanical perturbation on the growth of plants with special reference to anatomical changes, the role of ethylene, and interaction with other environmental stresses. *In* H Mussell, R Staples, eds, Stress Physiology of Crop Plants. John Wiley & Sons, New York, pp 25–29

Jarillo JA, Leyva A, Salinas J, Mart'nez-Zapater JM (1993) Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Arabidopsis thaliana*, a chilling-tolerant plant. Plant Physiol **101:** 833–837

Klee CB, Vanaman TC (1982) Calmodulin. Adv Protein Chem **35:** 213–322

Klüsener B, Boheim G, Liss H, Engelberth J, Weiler EW (1995) Gadolinium-sensitive, voltage-dependent calcium release channels in the endoplasmic reticulum of a higher plant mechanoreceptor organ. EMBO J **14:** 2708–2714

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature **352:** 524–526

Knight MR, Read ND, Campbell AK, Trewavas AJ (1993) Imaging calcium dynamics in living plants using semi-synthetic recombinant aequorins. J Cell Biol **121:** 83–90

Knight MR, Smith SM, Trewavas AJ (1992) Wind-induced plant motion immediately increases cytosolic calcium. Proc Natl Acad Sci USA **89:** 4967–4971

Marks MD, West J, Weeks DP (1987) The relatively large beta-tubulin gene family of *Arabidopsis* contains a member with an unusual transcribed 5′ noncoding sequence. Plant Mol Biol **10:** 91–104

Minorsky PV (1985) An heuristic hypothesis of chilling injury in plants: a role for calcium as the primary physiological transducer of injury. Plant Cell Environ **8:** 75–94

Minorsky PV (1989) Temperature sensing by plants: a review and hypothesis. Plant Cell Environ **12:** 119–135

Minorsky PV, Spanswick RM (1989) Electrophysiological evidence for a role for calcium in temperature sensing by roots of cucumber seedlings. Plant Cell Environ **12:** 137–143

Monroy AF, Dhindsa RS (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. Plant Cell **7:** 321–331

Monroy AF, Sarhan F, Dhindsa RS (1993) Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression. Evidence for a role of calcium. Plant Physiol **102:** 1227–1235

Mulqueen P, Tingey JM, De Horrocks W Jr (1985) Characterization of lanthanidine (III) ion binding to calmodulin using luminescence spectroscopy. Biochemistry **24:** 6639–6645

Peeters GA, Kohmoto O, Barry WH (1989) Detection of La^{3+} influx in ventricular cells by indo-1 fluorescence. Am J Physiol **256:** C351–C357

Pillai S, Bikle DD (1992) Lanthanum influx into cultured human keratinocytes: effect on calcium flux and terminal differentiation. J Cell Physiol **151:** 623–629

Powis DA, Clark CL, O'Brien KJ (1994) Lanthanum can be transported by the sodium-calcium exchange pathway and directly triggers catecholamine release from bovine chromaffin cells. Cell Calcium **16:** 377–390

Quiquampoix H, Ratcliffe RG, Ratkovic S, Vucinic Z (1990) ^{1}H and ^{31}P NMR investigation of gadolinium uptake in maize roots. J Inorg Biochem **38:** 265–275

Reid RJ, Smith FA (1992) Regulation of calcium influx in *Chara*. Effects of K^{+}, pH, metabolic inhibition, and calcium channel blockers. Plant Physiol **100:** 637–643

Roberts DM, Harmon AC (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 375–414

Roberts DM, Lukas TJ, Watterson DM (1986) Structure, function, and mechanisms of action of calmodulin. CRC Crit Rev Plant Sci **4:** 311–339

Schultz JE, Klumpp S (1982) Lanthanum dissociates calmodulin from the guanylate cyclase of the excitable ciliary membrane from *Paramecium*. FEMS Microbiol Lett **13:** 303–306

Segal J (1986) Lanthanum increases the rat thymocyte cytoplasmic free calcium concentration by enhancing calcium influx. Biochim Biophys Acta **886:** 267–271

Sistrunk ML, Antosiewicz DM, Purugganan MM, Braam J (1994) Arabidopsis *TCH3* encodes a novel Ca^{2+} binding protein and shows environmentally induced and tissue-specific regulation. Plant Cell **6:** 1553–1565

Thomashow MF (1994) *Arabidopsis thaliana* as a model for studying mechanisms of plant cold tolerance. *In* EM Meyerowitz, CR Somerville, eds, *Arabidopsis*. Cold Spring Harbor, Cold Spring Harbor, NY, pp 807–834

Tsien RY (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry **19:** 2396–2404

Valvekens D, Van Montagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci USA **85:** 5536–5540

Verwoerd TC, Dekker BM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res **17:** 2362

Weast RC, ed (1986) CRC Handbook of Chemistry and Physics. CRC, Boca Raton, FL

Weihe E, Haartschuh W, Metz J, Bruhl U (1977) The use of ionic lanthanum as a diffusion tracer and as a marker of calcium binding sites. Cell Tissue Res **178:** 285–302

Wendt-Gallitelli MF, Isenberg G (1985) Extra- and intra-cellular lanthanum: modified calcium distribution, inward currents and contractility in guinea pig ventricular preparations. Pfluegers Arch **405:** 310–322

White TC, Simmonds D, Donaldson P, Singh J (1994) Regulation of *BN115*, a low-temperature-responsive gene from winter *Brassica napus*. Plant Physiol **106:** 917–928

Xu W, Purugganan MM, Polisensky DH, Antosiewicz DM, Fry SC, Braam J (1995) Arabidopsis *TCH4*, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. Plant Cell **7:** 1555–1567

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251–264

Zimmerman UP, Schlaeffer WW (1988) Activation of calpain I and calpain II: a comparative study using terbium as a fluorescent probe for calcium-binding sites. Arch Biochem Biophys **266:** 462–469